

RAPID ASSEMBLY OF A WOUND PLUG: STAGE ONE OF A TWO-STAGE WOUND REPAIR MECHANISM IN THE GIANT UNICELLULAR CHLOROPHYTE *DASYCLADUS VERMICULARIS* (CHLOROPHYCEAE)¹

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Upon injury, selected coenocytic algae are capable of forming temporary wound plugs to prevent detrimental cytoplasmic loss. Wound plugs of *Dasycladus vermicularis* ([Scropoli] Krasser) were harvested 5 min post-injury and dried. The plug material contained 94% water and can be considered a hydrogel. The gel plug extended several millimeters from the cut end and filled the space inside the cell wall, which resulted from cytoplasmic retraction. Total organic carbon included 55% sugars, 5%–15% protein, and 0.18% lipids. The major sugars were glucose, galactose, mannose, and galacturonic acid. Fluorescein isothiocyanate-lectins specific for these sugars were localized around the plug matrix. Sulfur content calculated as sulfate corresponded to 17% of the carbohydrate by weight, and sulfated material was detected in plugs by Alcian Blue staining. Formation of the initial plug occurred within 1 min of injury and was not significantly perturbed by the addition of ionic, antioxidant, or chelating agents to the seawater medium. However, addition of exogenous D(+)-galactose and D(+)-glucose prevented formation of the nascent gel plug. Wound plugs that were allowed to form from 10 min up until 24 h post-injury were isolated and incubated with selected biochemical probes to identify the biochemical processes involved in plug formation. The operative strategy in *Dasycladus* to prevent “cytoplasmic hemorrhage” required availability of sequestered carbohydrate and lectin precursor components throughout the thallus for plug assembly. Once the initial assembly had commenced, additional biochemical interactions were initiated (as a function of time) to promote structural integrity.

Key index words: algae; carbohydrate; coumarin; *Dasycladus vermicularis*; gelling, lectin; wounding

Abbreviations: FITC, fluorescein isothiocyanate; IC₅₀, 50% inhibitory concentration

Siphonalean green algae (Dasycladales and Caulerpaceles) exhibit a unique ability to cope with injury by rapidly forming gel-like wound plugs. Upon injury, a physiological response is initiated that subsequently leads to the immediate sealing of the wounded area by exuding intracellular contents. Under the protection of this temporary gelatinous plug, the intricate regeneration of a new cell wall may commence.

We propose a repair mechanism in which a wound plug is rapidly assembled, first immediately by lectin-carbohydrate interactions and second by the synergistic effects of hydrophobic interactions, metal chelation, and hydrogen bonding.

Interesting wound responses have been observed in a variety of marine algae (*Bryopsis* spp., *Cladophoropsis membranacea* (Ag.) Boerg., and *Ernodesmis verticillata* (Kütz.) Boerg.). Upon injury, cytoplasmic material exuded into the surrounding seawater and formed protoplasts initially by an agglutination process (Tatewaki and Nagata 1970, La Claire 1982a,b, Kobayashi and Kanaizuka 1985, Pak et al. 1991, Kim et al. 2001). It is believed that naturally occurring adhesive materials are present in these algal cells and that upon injury aggregation is directed by the sequential action of acidic vacuolar sap and alkaline seawater (Pak et al. 1991). More specifically, evidence has also been reported that lectin-based wound healing responses are evident. Repair cells of *Antithamnion nipponicum* (Kim et al. 1995) and *Antithamnion sparsum* (Kim and Fritz 1993) have been shown to produce a carbohydrate-rich substance that specifically binds concanavalin A. This specificity indicates that the wound healing substance contains α -D-mannosyl and/or α -D-glucosyl residues at sterically accessible positions.

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The repair mechanism in siphonaeal algae has been characterized as having at least six distinct steps (Menzel 1988). However, in retrospect it seems feasible to reduce this number down to two generalized themes: 1) extrusion of plug material that gels as a preliminary barrier against the external medium (accompanied with a retraction of the cytoplasm from the wounded area) and 2) hardening of the gel into a rigid protective matrix. This matrix has been hypothesized to solidify as a function of time by a peroxidase-mediated cross-linking process involving endemic catecholic molecules (Menzel et al. 1983, Perez-Rodriguez et al. 2001, Ross and Jacobs 2003). The focus of this current study is to present a detailed description of the first step: the formation of a wound plug by the gelling of plug precursor material in *Dasycladus vermicularis*.

We investigated the wound healing process as well as the adhesive components in plug formation using biomolecular detection systems, adhesive microspheres, biochemical probes, and anti-biotin fluorescein isothiocyanate (FITC) conjugates. Our new evidence supports the hypothesis that the initial wound repair mechanism is based on a rapid gelling process (less than a minute) that exploits a complementary lectin-carbohydrate ligand system. From 10 to 30 min post-injury, other biochemical interactions are initiated that help establish the integrity of the wound plug. We provide evidence that this initial gelling (seen from 1 to 30 min post-injury) is the first step of an efficient and vital repair mechanism.

MATERIALS AND METHODS

Algal material. Juvenile and adult specimens of *D. vermicularis* (Scropoli) Krasser were collected live near Indian Key, Florida (USA) and Cay Sal Bank, Bahamas and either cultured or flash frozen with liquid N₂ in spring 2002. For laboratory studies a unialgal culture (strain, LB 2685 *D. vermicularis*) was obtained from the Culture Collection of Algae at the University of Texas at Austin (USA). Cultures were maintained using a 12:12-h light:dark photoperiod (at a photon flux density of 50–60 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and at 25° C). Cultures were grown in Muller's medium (Berger and Kaever 1992) that was filtered through 0.22- μm membranes (no. SCGPT10RE, Millipore, Bedford, MA, USA) before introduction of the algae.

Carbon, hydrogen, nitrogen, and sulfur analysis. Determination of the elemental composition (weight percent) of organic material in lyophilized wound plugs was conducted at the Marine Science Institute Analytical Laboratory (University of California, Santa Barbara) and by Quantitative Technologies Inc. (Whitehouse, NJ, USA). Briefly, freshly formed plugs were isolated and collected with microforceps and subsequently pooled in deionized water. Samples were vortexed, sonicated, and centrifuged. The supernatant was discarded (to remove as much residual salts from seawater as possible). This process was repeated several times. Pooled plugs were then flash frozen with liquid N₂ and lyophilized overnight. One milligram of lyophilized product was submitted for each sample analysis. Carbon, hydrogen, and nitrogen were determined using a 2400 CHN Elemental Analyzer (Perkin-Elmer, Norwalk, CT, USA). Sulfur was determined by first combusting the sample in an oxygen-rich environment to convert the covalently bound sulfur to the anion sulfate. The

anions were then trapped in a quench solution that was analyzed by a titrating with barium perchlorate in the presence of meso-2,3-dimercaptosuccinic acid III indicator.

Major sugar determination. Freshly formed plugs were isolated, collected, and treated as described. One milligram of crude plug was weighed out and added to 0.5 mL of 12 M H₂SO₄. The sample was sonicated for 15 min at 60° C. Four and one-half milliliters of milli-Q water (Millipore) was added for a total volume of 5 mL. Samples were then sealed in ampules and hydrolyzed for 20 h at 100° C. Hydrolysis was terminated by placing the samples on ice for 30 min. Samples were then neutralized by titrating (in 1-mL increments) into a 30-mL Oak Ridge tube (Fisher Scientific, Atlanta, GA, USA) containing 0.72 g of combusted CaCO₃ until effervescence ceased. Samples were then sonicated for an additional 15 min at 60° C. Samples were centrifuged for 30 min at 14,000 rpm. Approximately 3.2 mL of supernatant was collected and subsequently filtered through 0.22- μm 13-mm polycarbonate filters. Samples were run on a Bio-LC pulsed amperometric detection system (Dionex, Sunnyvale, CA, USA) with a CarboPac PA10 (250 \times 4 mm) column (Dionex). Comparisons were made using authentic standards.

Protein content determination. Freshly formed plugs were permitted to form for up to 10 min post-injury. Plugs were detached from the thallus with microforceps and pooled in deionized water in a microcentrifuge tube. Plugs were processed as described above.

Lyophilized plugs (0.5 mg) were hydrolyzed *in vacuo* with 300 μL of 6 N HCl and 20 μL of phenol overnight at 120° C. Hydrolysates were flash evaporated at 55° C and washed with 500 μL each of deionized water, methanol, and deionized water. The final residue was resuspended in 40–100 μL of sample buffer before loading and running on a 6300 autoanalyzer (Beckman, Fullerton, CA, USA), which has a dual wavelength ninhydrin-based detection system.

Total lipid determination. Wound plugs were collected as described above. Lipids were isolated according to the method of Bligh and Dyer (1959). Lipids were extracted from gelled plugs using CHCl₃:MeOH (2:1). All solvents used were HPLC grade. Extractions were done in 45-mL Corex II (Fisher Scientific) glass centrifuge tubes with Teflon-lined caps. Tubes were inverted for 3 min and then centrifuged at 1000g for 10 min on a TJ-6 Beckman centrifuge at 4° C. The organic layer was separated, and the aqueous layer was re-extracted. All organic phases were pooled and reduced *in vacuo*.

Fluorescent microsphere assay. To better understand the adhesive properties of the gelled material as a function of time (post-injury), a fluorescent microsphere solution was prepared by the addition of one drop of stock (YG, 1 μM , Polysciences Inc., Warrington, PA, USA) solution to 300 μL deionized water just before use. This secondary stock dilution was further diluted 1:10 in artificial seawater to make the working reaction mix.

Algal cells were injured via a complete mid-thallus cut in 2 mL of artificial seawater on a depression slide. At selected time points ($t = 0, 10, 20, 30$, and 40 min post-injury) the seawater was drawn off and replaced with 2 mL of the microsphere reaction mix. Each experimental sample was mixed for 1 min on a rotary shaker. The reaction mix was then drawn off, and the cells were washed in artificial seawater and subsequently viewed via fluorescence microscopy. The specimens were examined using a universal microscope (Zeiss, Jena, Germany) in conjunction with the Pixera 120es Application suite photo program (Pixera Corporation, Los Gatos, CA, USA). A broad-emission FITC filter set (450–490 nm excitation and >520 nm emission filter, Zeiss) was used.

Carbohydrate localization experiments. Algal cells were injured via a mid-thallus cut on a glass slide and immediately

placed in 5 mL seawater. Biotinylated lectins (concanavalin A, *Ricinus communis* agglutinin I, peanut agglutinin, *Dolichos biflorus* agglutinin, *Ulex europaeus* agglutinin I, and soybean agglutinin, Vector Laboratories, Burlingame, CA, USA) were added ($10 \mu\text{g} \cdot \text{mL}^{-1}$) to the reaction mix and allowed to incubate on a shaker for several minutes. Algal thalli were subsequently washed several times in seawater. As a second step, a monoclonal anti-biotin FITC conjugate (Clone BN-34, Sigma, St. Louis, MO, USA) was added (1:160) to permit detection by fluorescence microscopy (Elborough et al. 1996, Cooper et al. 1997). Controls were conducted by competing off bound lectin with the appropriate complimentary monosaccharide. The specimens were viewed via fluorescent microscopy as described above.

Alcian blue staining. Alcian blue 8GX (a histological stain for sulfated polysaccharides) was mixed with artificial seawater ($8 \text{ mg} \cdot \text{mL}^{-1}$). After vortexing, the mixture was centrifuged for 10 min at $12,000g$. The supernatant was collected and added to wounded algae at selected time points and allowed to incubate for several minutes. At this point the algal specimens were washed several times with fresh seawater.

Carbohydrate inhibition experiments. The carbohydrates used in this study are listed in Table 2. Algal cells were injured via a complete mid-thallus cut on a glass slide and immediately placed in the carbohydrate-rich modified seawater. Five minutes post-injury the algal cell was examined for its ability/inability to form a wound plug. Each experiment included 10 samples to determine a percentage of 10 injured cells that formed wound plugs in the selected media. Each experiment was run three for a total of 30 samples.

Effects of modified seawater on wound plug formation. To examine the effects of modified seawater on the wound healing response in *D. vermicularis*, either filtered seawater, an adapted form of Muller's medium (Berger and Kaever 1992), or a very basic synthetic seawater recipe was used. The benefit of the basic synthetic recipe was to evaluate the effect of omitting specific ions on algal wound healing abilities. The basic synthetic media consisted of 450 mM NaCl, 30 mM MgCl_2 , 16 mM MgSO_4 , 9 mM CaCl_2 , 10 mM KCl, and 10 mM Tris-HCl (pH 7.8).

Plugs were permitted to form for 2, 10, 30, 40, and 80 min post-injury in artificial seawater. At this point, plugs were either examined in their natural state (attached to the algal thallus) or isolated from the injured thallus and stained with toluidine blue (T 3260, $1 \text{ mg} \cdot \text{mL}^{-1}$ dissolved in artificial seawater [pH 5.0], Sigma). At this pH, toluidine blue stains the plug's acidic polysaccharide components a purple-pink color. This permits visualization of the plugs that may otherwise exist as a colorless material. Plugs were incubated with selected biochemical agents to identify the types of chemical interactions partaking in plug formation as a function of time. At selected times points (post-injury) 1 mL of disrupting agent (i.e. EDTA, 0.1% Triton-X, etc. dissolved in artificial seawater) was added to the plug and permitted to mix on a rotary table for 5–10 min. Ascorbic acid, 2-mercaptoethanol, Tween 20, Triton X-100, urea, guanidine hydrochloride, sodium azide, EDTA, EGTA, potassium bromide, heparin, and bovine liver catalase were purchased from Sigma Inc. (St. Louis, MO, USA).

RESULTS

Wound plug formation is an efficient repair mechanism vital to the survival of *D. vermicularis*. Upon initial injury of the cell wall, the change in turgor pressure alone accounts for the explosive extrusion of internal vacuolar and cytoplasmic contents. After several seconds, a more controlled extrusion is observed. This extrusion slows in velocity until about 1 to

2 min post-injury when the internal contents have actually gelled in place at the site of injury, forming a protective barrier. This barrier serves to prevent further cytoplasmic loss and to block the invasion of foreign pathogens.

Carbon, hydrogen, nitrogen, and sulfur analysis. Elemental composition (weight percent) of pooled wound plugs showed an organic carbon composition of 31.06% (Table 1). The total organic carbon (31.06% weight) was subdivided into protein (5%), major sugars (54.6%), and free fatty acids (0.18%). Automated amino acid analysis indicated that overall amino acid levels could vary from 5% to 10% of plug material. The contribution of nitrogen in a protein with predominantly neutral residues indicates that N levels measured by CHN analysis should correspond to protein levels of 15%. The differences in estimates of percent protein may be due to non-amino acid nitrogen components. These compounds may include amino sugars, polyamines, and amine containing groups in lipids (i.e. ethanolamine, choline etc) that have not yet been speciated.

In the sulfated polysaccharides of wound plugs, sulfate predominantly exists as SO_3^- (the fourth oxygen is actually the linkage with the sugar, replacing an OH). Elemental sulfur comprises 0.4% of the combined SO_3 atomic mass (FW 80), which means SO_3 represents 1.42% of the dry mass of the plug. Major sugars represented 54.6% of the total organic carbon or 8.01% of the dry weight of the wound plug. The dry mass of total SO_3 divided by the dry mass of the major carbohydrates ($1.42/8.01$) yields the percentage of sulfated carbohydrates (approximately 17.7% of major sugars detected are sulfated). This is very close to the published results for related algal polymers (Haug 1976, Lahaye and Axelos 1993, Lahaye et al. 1996). This number is not an exact value because hydrogen mass was not considered in the sulfate mass calculations and sulfur could originate from methionine or cysteine (however, because these amino acid levels were only detected in trace amounts, they were neglected).

TABLE 1. (A) Percent abundance of carbon, hydrogen, nitrogen, and sulfur in wound plugs from *Dasycladus vermicularis* and (B) distribution of organic carbon in wound plugs from *D. vermicularis*.

(A) Elemental analysis					
Plug weight (μg)	%C	%H	%N	C/N	%S
1085	31.06	4.63	2.31	13.99	0.51
(B) Breakdown of organic carbon					
Components	Percent (\pm SE)				
Protein	5 ± 1.20				
Major sugars	54.6 ± 5.18				
Galactose	12.5 ± 0.61				
Glucose	26.7 ± 1.73				
Mannose	8.3 ± 0.96				
D-Galacturonic acid	7.1 ± 0.39				
Free fatty acids	0.18 ± 0.04				

Major sugar determination. For an analytical analysis of major sugars, lyophilized plugs were acid hydrolyzed and run on a Dionex Bio-LC HPLC. Major sugars represent 54.6% of the total organic carbon. As shown in Figure 1, the predominant hydrolysate component was glucose (representing 50% of the neutral sugars). Although starch grains could account for the abundance of glucose, only the overall plug composition was analyzed. Also present in significant concentrations were galactosyl (23% of the total neutral sugars), mannosyl (15%), and galacturonic acid (13%) residues. Trace sugar representatives included rhamnose, fructose, and arabinose.

Protein content determination. Protein was detected as a constituent in 5-min gelled plugs (post-injury). Based on amino acid analysis, protein represents 5%–8% mass of lyophilized plugs. Amino acid profiles for hydrolyzed wound plugs showed Gly (13%–20% [mol%]), Ala (5%–17%), Val (8%–20%), Ser (10%–13%), and Asp/Asn (13%–22%) as the major amino acid constituents. Glucose and Asp could not be differentiated from Gln and Asn, respectively.

Amino acid percent composition varied from sample to sample, most likely because of the random nature of what happens to gel within the plug matrix during a specific injury event. For example, certain plugs may have a higher content of chloroplasts or other organelles, whereas another plug may have a higher concentration of cytoplasmic or vacuolar fluid. Depending on the plug constituents, proteins (hence amino acids) will vary in concentration from plug to plug.

Fluorescent microsphere assay. The binding of fluorescent microspheres was used to visualize the adhesive properties of gelling exudate in wounded areas (Fig. 2, a–d). The mechanism of microsphere binding seen in these studies has not been completely defined at the molecular level. The polystyrene spheres represent a high surface area for polymer binding that appears to be based on nonspecific electrostatic interactions. The microspheres have been noted to

bind to a variety of biological surfaces (Vreeland et al. 1993, Hable and Kropf 1998) that are adhesive or viscous in their physical properties.

When microspheres were added at the time of injury ($t = 0$), the explosive extrusion of intracellular contents forced the spheres to disperse throughout the reaction mixture (Fig. 2a). When injury first occurred in *D. vermicularis*, the turgor change resulted in an explosive extrusion of vacuolar contents. After several seconds, a more controlled extrusion was observed that occurred at the same time as the retraction of the cytoplasm away from the wound site.

At 10 min post-injury, a defined plug could be outlined by the fluorescent spheres (Fig. 2b). The adhesive capabilities of the gel material could be detected up to 30 min post-injury. At this point the external adhesive capability was diminished (Fig. 2d).

Carbohydrate localization experiments. Carbohydrate localization on the plug surface was verified by the addition of selected biotinylated lectins with a monoclonal anti-biotin FITC conjugate. Concanavalin A and *Ricinus communis* agglutinin I showed the strongest labeling specificity for plugs 10 min post-injury. This is in accordance with the fact that their respective ligand sugars (concanavalin A, glucose and mannose; *R. communis* agglutinin, galactose and glucosamine) are in high abundance in plug material (Fig. 2, e–h). If the same lectins are premixed with injured algae and their respective complementary exogenous sugars, the fluorescent labeling of the plug is greatly diminished. When plugs were labeled with concanavalin A and/or *R. communis* agglutinin I after 40 min post-injury, the fluorescent labeling was diminished, indicating that perhaps cross-linking (stage II) prevents lectin binding.

The utilization of *Ulex europaeus* agglutinin I (specificity toward α -linked fucose residues) showed slight labeling specificity for plugs (10 min post-injury). Peanut agglutinin (β -1,3-*N*-acetylgalactosamine specificity), soybean agglutinin (structures with terminal

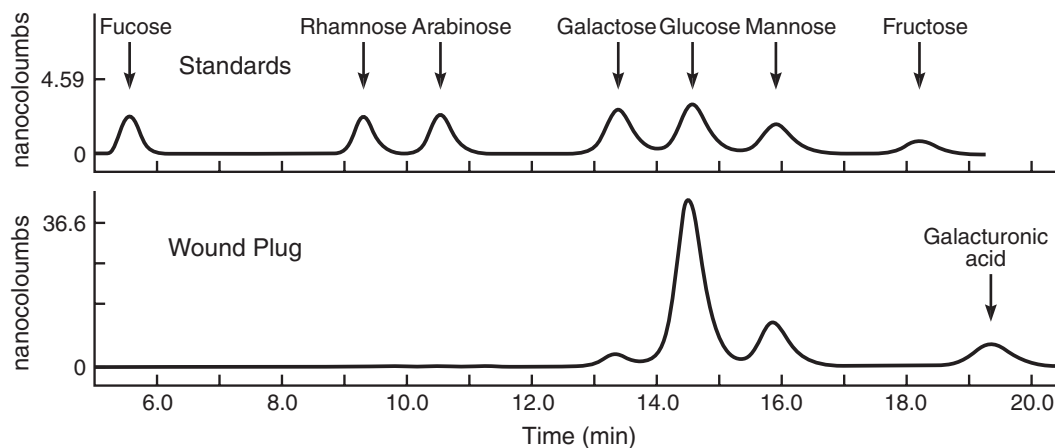


FIG. 1. Major sugar analysis. Standards (100 nM) and wound plug profile.

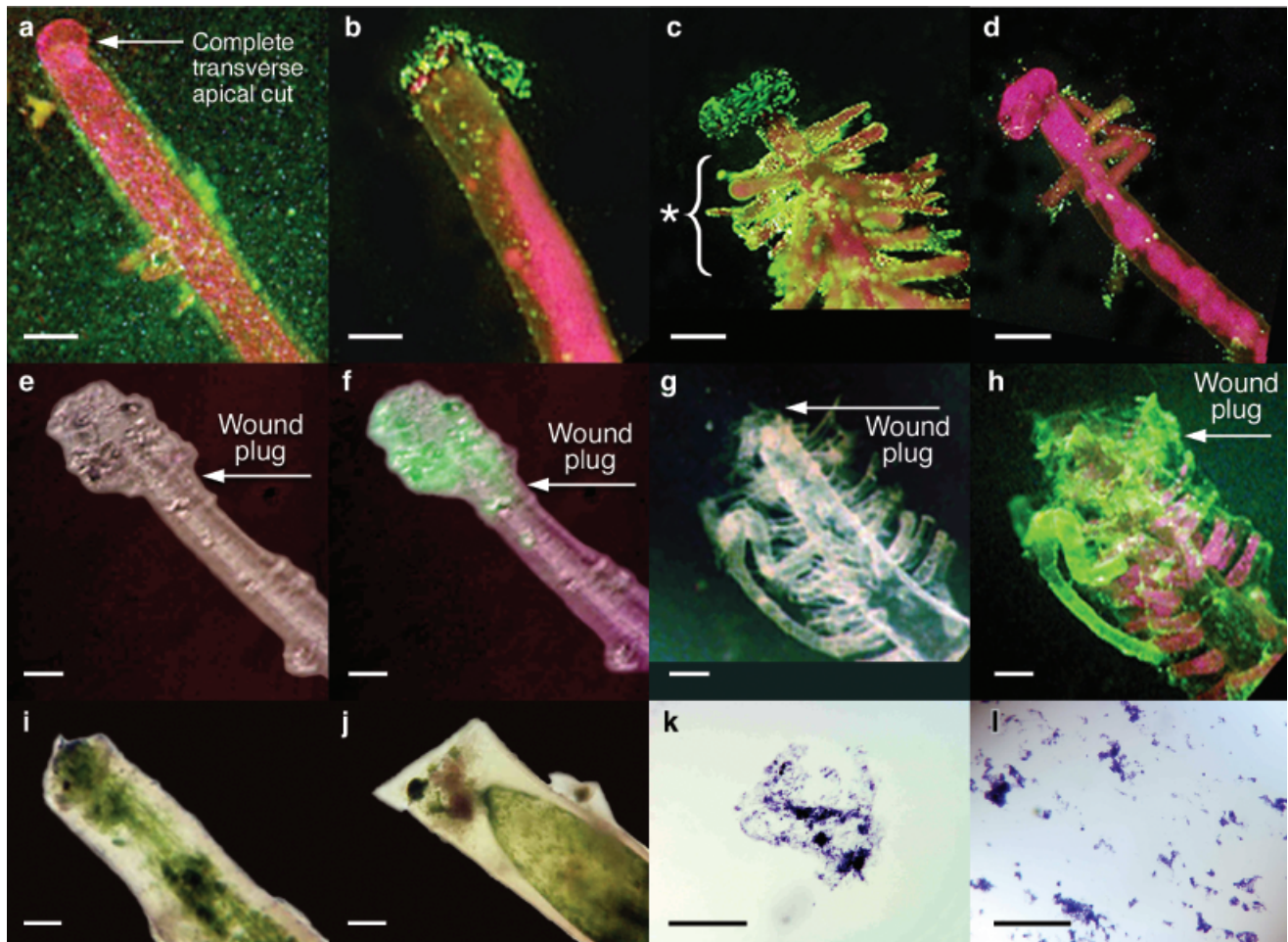


FIG. 2. (a–d) Examination of “stickiness” of plug via yellow fluorescent microspheres (YG, 1 μ M, Polysciences Inc.). Chl is localized in red. (a) Addition of microspheres immediately upon injury. No localization because cytoplasmic contents are dispersed throughout media. (b) 10 min post-injury. Microspheres locally adhering to plug region. (c) 20 min post-injury. Microspheres adhering to plug region. (Note: Some microspheres are adhered to branchlets because plug precursor material may have gotten caught there upon injury.) (d) 30 min post-injury. Decrease in adhesion capabilities of microspheres to plug. Scale bar, 8 mm. *Internal cytoplasmic contents that exuded from initial injury sight that became caught up on branchlet tips. (e–h) Carbohydrate localization experiment. Algal cells were injured via a mid-thallus cut on a glass slide and immediately placed in 5 mL seawater for 10 min, allowing adequate time for plug formation. e and f show concanavalin A binding (to glucose and mannose residues). (e) Visible light; (f) visible and UV light. g and h show *Ricinus communis* agglutinin I binding (to galactose, D-glucosamine residues). Scale bar, 4 mm. (i–j) Exogenous carbohydrate inhibition of plug formation (5 min post-injury). (i) Control: natural plug formation in artificial seawater. (j) Wounding algae in artificial seawater with 100 mM D(+)-galactose. Scale bar, 4 mm. (k–l) Toluidine blue staining of isolated wound plugs. (k) Wound plug collected 10 min post-injury and stained with toluidine blue. Scale bar, 4 mm. (l) Wound plug, collected 10 min post-injury, stained with toluidine blue, and immediately incubated with 100 mM EDTA, 250 mM glucose, 250 mM galactose, 0.1% Triton X-100, and 6 M guanidine hydrochloride (or 6 M urea). Dissociation of the plug was observed. Scale bar, 4 mm.

α - or β -linked *N*-acetylgalactosamine specificity), and *Dolichos biflorus* agglutinin (*N*-acetylgalactosamine specificity) showed very little labeling specificity.

Alcian blue staining. The participation of charged sugars (sulfated polysaccharides at seawater molarity) representing a component of wound plugs was detected histologically by Alcian blue 8GX (data not shown). This cationic dye binds to algal acidic carbohydrates and glycosaminoglycans by forming reversible electrostatic bonds between the cationic dye and the anionic sites on the polysaccharide. Although Alcian blue staining is usually conducted under acidic conditions, our results showed the presence of sul-

fated polysaccharides in artificial seawater ranging from pH 5 to 8. The formation of Alcian blue gel wound-associated patches can occur from several minutes through 24 h post-injury, with constituent variability probably due to chance differences in concentration and composition of different plug precursor content (i.e. carbohydrate vs. phenolic concentrations).

Carbohydrate inhibition experiments. To determine whether the initial wound plug formation was based on a lectin–carbohydrate interaction, individual algae were injured in artificial seawater containing selected concentrations of exogenous sugars. Five minutes

TABLE 2. Carbohydrates used with associated IC_{50} with SE.

Carbohydrate	IC_{50} (mM)
D(+)-Glucose	109 \pm 3.22
D(+)-Glucosamine	190 \pm 4.93
D(+)-Galactose	74 \pm 1.77
D(+)-Galactosamine	170 \pm 3.33
D-Glucuronic acid	175 \pm 6.01
D-Galacturonic acid	175 \pm 2.89
D(+)-Mannose	173 \pm 1.45
Fucoidan	175 \pm 2.89
D(-)-Ribose	> 500
Maltose	> 500
D(-)-Fructose	> 500
D(+)-Xylose	> 500
Sucrose	> 500
α -Lactose	> 500
D(-)-Arabinose	> 500
Xylan	> 500
Carrageenan iota	> 500
N-Acetyl-D-glucosamine	> 500

D(+)-Galactose and D(+)-glucose displayed the most selective activity against wound plug formation.

post-injury, the algal cell was examined for its ability/inability to form a wound plug. By adding selected exogenous sugars upon initial injury, gelling may be prevented. Table 2 lists the carbohydrates used with their associated 50% inhibitory concentrations (IC_{50}). The inhibitory concentration is the concentration at which an antagonist exerts its half-maximal effect on a ligand. The more selective an antagonist, the lower the IC_{50} . D(+)-Galactose had the lowest IC_{50} of 74 mM (Fig. 3), followed by D(+)-glucose with an IC_{50} of 109 mM. Both of these sugars were noted to be present in higher concentrations than other sugars identified from wound plugs (Fig. 1). D(+)-Glucosamine, D(+)-galactosamine, D(+)-mannose, and fucoidan were less selective in inhibition of plug formation (IC_{50} , 170–190 mM). Uronic acid inhibition (D-galacturonic acid and D-glucuronic acid with IC_{50} of 175 mM each) supports the likelihood that the carbohydrate plug component is a mixed uronate-neutral sugar polymer similar to fucoidan (IC_{50} , 175 mM). D(-)-Ribose, altose, D(-)-fructose, D(+)-xylose, sucrose, α -lactose, D(-)-arabinose, xylan, carrageenan iota, and N-acetyl-D-glucosamine were all nonactive in wound plug inhibition (IC_{50} > 500 mM).

Immediate gelling (up to 2 min post-injury). The initial gelling usually occurred within the first minute post-injury. The early gel material is a sticky substance that may easily be dislodged from the injured cell wall region simply by probing the gel with a pipette tip. Early gels (formed under 10 min post-injury) were difficult to collect. Manipulating the gels with micro-forceps resulted in the material simply adhering to the forceps as a syrupy substance.

Initial gelling was evaluated under a variety of experimental treatments. Modifying the pH (range, 3–10), ionic balance (half-strength and double-strength seawater), or omitting calcium or sulfate ions had no

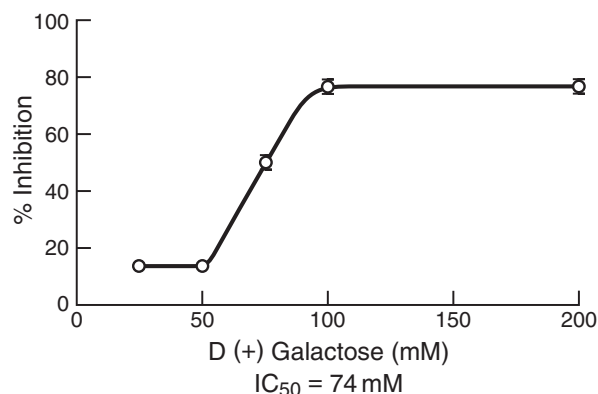


FIG. 3. Dose-response curve of D(+)-galactose (most efficient inhibitor of plug formation).

effect on the initial gelling. Furthermore, adjusting the redox potential (adding exogenous 2-mercaptoethanol [0.1%], H_2O_2 [5 mM], or ascorbate [5 mM]) had no effect on this process either. Even the addition of 100 mM sodium azide or 200 U catalase had no inhibitory effect on the gelling process. The addition of 0.1%–0.5% nonionic detergents (Triton X-100 or Tween 20) or ionic detergents (0.1%–0.5% SDS) had no effect on the immediate plug forming process. The addition of chaotropes, which disrupt hydrogen bonding (6 M guanidine hydrochloride or 6 M urea), had no effect on immediate plug formation when added on an isolated or combined basis (incubation times ranged from 1 to 20 min; mixing on a rotary table).

Young plugs (formed 1–2 min post-injury) were isolated from the algal thallus and placed in a microtube of seawater. Plug morphology could be visualized with the addition of toluidine blue (staining sulfated polyglycans). The immediate addition of 250 mM glucose + 250 mM galactose, followed by a 5-min incubation period, yielded total dissociation of the plug. Dissociation of the wound plug in the intact algae is shown in Figure 2, i and j. The addition of 250 U heparin, only in addition to 1) 100 mM EDTA (metal chelator) and 6 M chaotrope or 2) 6 M chaotrope and 0.1% Triton X-100, showed slight dissociation of the plug. It appears that the initial gelling process (0–5 min post-injury) was based on a lectin-carbohydrate interaction that could be competitively inhibited with the addition of D(+)-glucose and D(+)-galactose.

Gelling (10 min post-injury). Gels that were allowed to solidify for 10 min (Fig. 2k) were actually rigid enough to maintain a distinct shape even when manipulated and separated from the injured cell wall. If plugs were permitted to form for 10 min post-injury, the addition of exogenous sugars resulted in no visible breakdown of the plug matrix. At this time point it seemed as though a combinatorial event took place where several other interactions commenced to promote the integrity of the wound plug. The same results were observed for plugs that were allowed to mature up to 30 min post-injury.

If plugs were incubated with 6 M guanidine hydrochloride or 6 M urea, they would not dissociate. If plugs were incubated with 100 mM EDTA or 0.1% Triton X-100, they would not dissociate. Only the addition of heparin (sulfated polysaccharide, which may assist in the dissociation of the plug by a competitive inhibitory process) in conjunction with 0.1% Triton X-100 yielded a mild dissociating effect. If plugs were incubated with either 6 M chaotrope and 0.1% Triton X-100 or 6 M chaotrope and 100 mM EDTA, slight dissociation of the plug was visible (data not shown).

If plugs were isolated (10 min post-injury) and incubated with 100 mM EDTA, 250 mM glucose, and 250 mM galactose, 0.1% Triton X-100, and 6 M guanidine hydrochloride (or 6 M urea), then dissociation of the plug was observed (Fig. 2, k and l).

When any of these agents was omitted from the reaction mixture, the plug was still observed in an intact state, indicating that multiple types of reactive sites are used in the plug-forming process.

Gelling (40 min post-injury). Plugs continue to harden as a function of time. At 35–40 min post-injury the plugs turned brown, which can be attributed to the oxidative cross-linking of plug constituents (Ross and Jacobs 2003). Only the combined addition of 250 mM galactose, 250 mM glucose, 100 mM EDTA, 0.1% Triton X-100, 250 U heparin, 6 M chaotrope results in a moderate (not total) dissociation of the plug matrix. Even though (at 40 min) the cross-linking commenced, these interactions are not mature enough (or at least not enough of them have formed) to prevent plug dissociation by all the agents mentioned above. By 80 min post-injury, the above-mentioned agents had no effect on the disruption of the plug matrix.

DISCUSSION

Early events after cellular injury in *D. vermicularis* include retraction of the cytoplasm and the assembly of a soft, adhesive, gel-like plug (Menzel 1980). The gel plug fills the space inside the cell wall produced by the cytoplasmic retraction; it also extends several millimeters into the surrounding seawater. Upon initial injury, the cytoplasm of *D. vermicularis* retracts for a distance varying from 10% to 50% of the total cell length. This contraction is similar in nature to the actin-based protoplasmic contraction reported in *Boodlea* sp. upon injury (La Claire 1982a,b, 1984).

When injury first occurs in *D. vermicularis*, the turgor change results in an explosive extrusion of vacuolar contents. After several seconds, a more controlled extrusion is observed that occurs at the same time as the retraction of the cytoplasm away from the wound site. The turgor pressure alone most likely accounts for the brief extrusion of plug material beyond the cut surface. In *Caulerpa* and *Acetabularia* the turgor pressure is relatively low, 40–50 mOsm · kg⁻¹ and 2.2–2.6 bar, respectively. Because of these low values, the extrusion does not continue for more than a minute (Kirst and Bisson 1979, Wendler et al. 1983).

Reliance on an extensive three-dimensional network of lectin–polysaccharide interactions to form the primary plug after damage to the cell wall in *Dasycladus* is reminiscent of the action of von Willebrand factor in the cell-mediated stasis after injury to animal tissues. The von Willebrand factor is a multidomain protein, each domain of which has a binding target. Chief among these are the D-domains, which bind glycosaminoglycans and help to seal off the site of injury (Ruggeri 2001).

Polysaccharides, glycoproteins, and proteoglycans have not only been shown to be a major component in wound plugs of coenocytic algae (Dreher et al. 1978, 1982, Menzel 1987), they also play a major role in the process of adhesion for many fouling diatoms and bacteria (Hoagland et al. 1993). The precise involvement of polysaccharides in adhesion remains elusive because of the natural complexity of their structures. Wustman (1997) hypothesized that the extracellular adhesives from the diatom *Achnanthes longipes* are a multicomponent composite made up of uronic acid and sulfate ester containing polysaccharides, making up the bulk of the adhesive and responsible for the gelling and flexibility characteristics, and proteins, glycoproteins, and/or phenolics involved in organization and assembly of the first two components. The same generalization may be made for the wound plugs of *D. vermicularis*.

We observed that carbohydrate represents the bulk of the total organic carbon (54%) of isolated wound plugs from *D. vermicularis*. Data assimilated from crude extracts of *Ulva* spp. showed that sugars represent a major constituent (~40% of organic carbon). This is in agreement with the reports from other members of the Ulvaceae family (Percival and McDowell 1967). Dawes and Goddard (1978) reported on average that wound plugs of *Caulerpa* spp. were represented by 35% dry weight total carbohydrate or 90% organic weight total carbohydrate. Predominating sugars were identified as glucose and galactose as seen in *D. vermicularis*. Calculations show that approximately 17% of this carbohydrate pool is sulfated. Similar sulfated polysaccharides have been reported to be involved in the gelling properties of *Ulva* spp. (Haug 1976, Lahaye and Axelos 1993, Lahaye et al. 1996), *Caulerpa* spp. (Dreher et al. 1982), *Codium* spp. (Percival and McDowell 1967) and have been found to be a component of the extracellular matrix involved in diatoms (Hoagland et al. 1993, Wustman et al. 1997). Additionally, ¹⁴C-labeling experiments have indeed shown evidence that sulfated carbohydrates are involved in the wound healing response in *Caulerpa* spp. (Hawthorne et al. 1981) and in *Udotea* (Mariani-Colombo and Decarli 1980). Sulfate bridges could clearly contribute to wound plug integrity as displayed in the bridging of sulfated galactans as reported by Rees (1969). Additionally, sulfated polysaccharides provide a negatively charged barrier that reduces the opening of Ca²⁺ channels in response to hypotonic shock and contribute to the steady-state turgor pressure by reducing the activity of water (Shepard and Beilby 1999, Shepard and Heslop 1999, Shepard et al. 2004).

Effects of modified seawater on gelling. Our results indicate that the initial gelling was not affected by modified seawater. Changes in pH, redox, and ionic strength were similar as to the reports shown by Dreher et al. (1982). However the addition of 2-mercaptoethanol or a chaotropic agent seemed to inhibit plug formation in selected *Caulerpa* species but not in *D. vermicularis*.

Cooksey (1981) found that removal of Ca^{2+} from artificial seawater not only inhibited the adhesive capabilities in selected diatoms but physically disrupted the integrity of the mucilage. In addition, the adhesive stalk polymers in *C. cistula* and *G. olivaceum* produce a sulfated xylogalactan that gels in the presence of Ca^{2+} . Kim et al. (2002) noted that the depletion of salts, magnesium, potassium, and sulfur from artificial seawater had little effect on the aggregation of cell organelles in *Microdictyon umbilicatum*; however, seawater void of calcium or magnesium promoted a decrease in organellar adhesion properties.

Contrary to these reports, the immediate wound healing ability in *D. vermicularis* was unobstructed by using synthetic seawater void of sulfate, calcium, magnesium, or potassium. The addition of EDTA (100 mM) partially inhibited plug formation in synthetic seawater only with the synergistic disassociating effects of other agents. However, EGTA in synthetic seawater, with concentrations up to 300 mM, had no effect on plug formation either alone or when added with other dissociating agents. This observation suggests that calcium is not required for efficient gelling.

Protein content determination. Conflicting reports exist on the levels and involvement of proteins in wound plug formation in siphonous algae. Studies on *Caulerpa* spp. (Dreher et al. 1982, Goddard and Dawes 1983) have not confirmed the involvement of proteinaceous material as an essential component of plug formation. However, in *Bryopsis hypnoides*, wounding was followed by the congregation of dense granular plug material with a notable amount of protein bodies (Burr and West 1971).

Trace amounts of cysteine is a comparable feature that was observed in protein extracts of *Ulva* spp. and *D. vermicularis*. There was no detection of hydroxyproline in wound plug hydrolysates, unlike the observations reported from protein fractions of other green algae (Thompson and Preston 1967). This rules out the involvement of hydroxyproline-rich proteins that have been commonly found as cross-linking components in volvicine algae and higher land plants.

Low levels of protein (1%–4% of dry weight) were noted in *Caulerpa* spp. (Dawes and Goddard 1978). Despite the low levels of protein detected, quality, not quantity, is the important issue. For example, external carbohydrate moieties have been regarded as specific markers for cell–cell recognition events in many cellular events. Such recognition systems depend on complementary binding between carbohydrate moieties of glycoconjugates on one cell with specific lectins on another cell (Kim et al. 1996, 2002).

In summary, we observed that the initial wound plug formed within 1 min post-injury was not significantly perturbed by the addition of ionic, antioxidant, or chelating agents to the seawater medium. However, addition of free glucose and galactose competitively inhibited the formation of the nascent gel plug. Plugs that were permitted to form for 10–30 min could not be disrupted strictly on the addition of these monomeric sugars. Dissociation of the plug could be observed with the simultaneous addition of a metal chelator, chaotropic agent, and non-ionic detergent in addition to the free sugars. This indicated that multiple types of reactive sites are used in the plug-forming process. By 40 min post-injury, it is believed that covalent interactions occur that add to the integrity of the plug. The covalent interactions result in hardening and the browning that has been hypothesized to result from the oxidative cross-linking of catechol molecules (Menzel et al. 1983, unpublished results). These interactions are believed to be initiated by an oxidative burst that supplies adequate amounts of H_2O_2 needed as a cosubstrate for a peroxidase-mediated cross-linking reaction of catecholic molecules.

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